EC50 for warfarin is 1.5 mg/L, and the apparent elimination half-life of prothrombin activity is 14 hours.

This approach can be applied quite generally to drug responses which are influenced by the time course of an intermediate substance whose kinetics are altered by a direct action of the drug.

Conclusion

The ability to predict drug effects *in vivo* has as its cornerstone the role of concentration linking drug to its receptor binding site. The consequence of binding may be expressed directly or indirectly in order to produce a drug response. The time course of drug concentrations *in vivo* is determined by the kinetics of drug distribution to the effect site and the kinetics of intermediates which express the final effect. Appreciation of the quantitative relations between time and concentration, and concentration and effect, can extend understanding of drug actions in man.

References

(1) Clark, A. J. (1933) The Mode of Action of Drugs on Cells. Arnold, London.
(2) Arunlakshana, O., Schild, H. O. (1959) Brit. J. Pharmacol. 14, 48–58.
(3) Holford, N. H. G., Sheiner, L. B. (1981) Clin. Pharmacokin. 6, 429–453.
(4) Gilman, A. G., Mayer, S. E., Melmon, K. L. (1980) in The Pharmacological Basis of Therapeutics (6th Edition) (Gilman, A. G., Goodman, L., Gilman, A., eds.) 32–33.
(5) Rosenbaum, J. S., Holford, N. H. G., Sadée, W. (1984) Brain Res., 317–324.
(6) Rosenbaum, J. S., Holford, N. H. G., Sadée, W. (1984) J. Pharmacol. Exp. Therap. (submitted).
(7) Sadée, W., Rosenbaum, J. S., Herz, A. (1982) J. Pharmacol. Exp. Therap. 223, 157–162.
(8) Holford, N. H. G., Guentert, T. W., Coates, P. C., Riegelman, S., Sheiner, L. B. (1981) Brit. J. Clin. Pharmacol. 11, 187–195.
(9) Kelman, A. W., Whiting, B. (1980) J. Pharmacokin. Biopharm. 8, 115–130.
(10) Holford, N. H. G., Sheiner, L. B. (1982) Pharmac. Ther. 16, 143–166.
(11) O'Reilly, R. A., Aggeler, P. M. (1968) Circulation 38, 169–177.

Nephelometric Immunoassay for Therapeutic Drug Level Monitoring

Takashi Nishikawa[1]

Abstract: Nephelometric immunoassay for the determination of drug levels in blood is based on the inhibition of immunoprecipitation by a hapten (drug). It represents a homogeneous method that does not require any separation steps nor radioisotopes. Precipitation in an aqueous solution can be quantitated by nephelometry (scattered light measurement) or turbidmetry (traversed light measurement). Advantages over other drug assay methods include its simplicity, speed and low cost. Only two reagents are added, and the subsequent reaction can be monitored optically with the potential for full automation. The reaction is usually completed in less than 15 minutes. The two reagents, anti-drug antibody and polyhaptenic antigen, can be easily prepared and are highly stable. Therefore, precipitation inhibition immunoassays and in particular nephelometric immunoassays are being commercially developed for routine therapeutic monitoring of drugs such as anticonvulsant drugs, aminoglycoside antibiotics and theophylline. The specificity is high, though depending on the cross-reactivity of the anti-drug antibody as is the case with other immunoassays. The sensitivity depends on a variety of factors such as antibody-hapten affinity, detection mode of the precipitation, and intrinsic turbidity of the test sample. But the sensitivity is sufficiently high for serum drug concentration greater than 1 µg/ml when less than 10 µl of serum are used. Variations of this assay technique include rate analysis for precipitate formation instead of endpoint analysis. Agglutination-, or particle aggregation-inhibition immunoassay is also a useful and more sensitive method. Finally, use of monoclonal antibodies can serve to enhance the specificity of nephelometric immunoassay of drugs.

Introduction

Therapeutic drug level monitoring (1) is widely performed in clinical laboratories for anticonvulsant drugs, cardiac glycosides, antiarrhythmics, aminoglycoside antibiotics, anticancer drugs, theophylline and lithium. Serum drug levels can serve as a guide to optimize dosage regimens for individual patients. A critical requirement for successful drug level monitoring is a reliable and accurate analytical method. For clinical use, the method should be simple, rapid, inexpensive, and require a small volume of blood. Therefore the clinical need for drug level data has stimulated development of novel analytical techniques.

Immunoassays and high performance liquid chromatography are now considered to be most suitable for routine therapeutic drug level monitoring. Each of these two methods has its own advantages and disadvantages. In general, immunoassay is more suitable for the analysis of a large number of samples than high performance liquid chromatography. Therefore various immunoassays have been applied to routine drug level monitoring such as radioimmunoassay, enzyme-labeled immunoassay and fluorescence-labeled immunoassay including fluorescence polarization immunoassay and fluorogenic substrate-labeled immunoassay. In this review, the nephelometric immunoassay for therapeutic drug level monitoring is described and discussed.

Assay Principle

Nephelometric immunoassay is based on the inhibition of precipitation by a hapten (Fig. 1). As Landsteiner described in his classical and pioneering studies (2), a hapten may be defined as a low molecular weight substance, too small to be immunogenic, but which can react with an antibody of appropriate specificity. When a small molecule is covalently conjugated with a large immunogenic protein and the conjugate is

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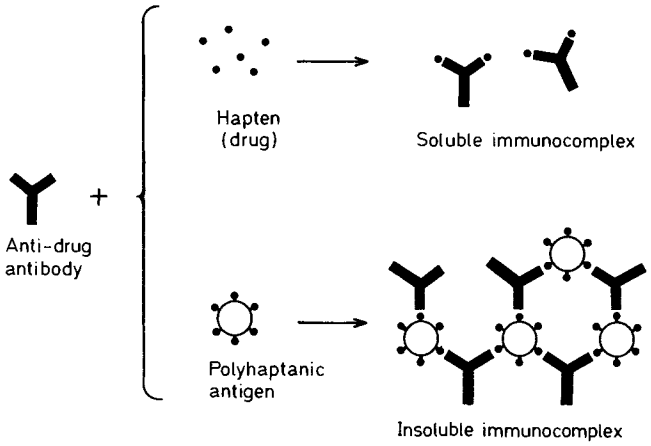

Fig. 1 Principle of the nephelometric immunoassay. Non-precipitating hapten (drug) inhibits the immunoprecipitation of the polyhaptenic antigen by competing for the binding site of the antibody with the polyhaptenic antigen.

injected into an animal, an antibody that specifically binds the small molecule is produced. The majority of drugs are organic substances of molecular weight less than 1,000 and, therefore, act immunologically as haptens. For example, carbamazepine (an anticonvulsant drug) is not immunogenic, but a conjugate of carbamazepine and bovine serum albumin is immunogenic when injected into a rabbit (Fig. 2). Because carbamazepine does not have a functional group to attach directly to protein, a butyrate group with a chemically active COOH is introduced. Approximately 20 carbamazepine molecules can be conjugated through the butyryl bridge with NH_2 groups in each albumin molecule. Various methods are available for conjugation of drugs or drug derivatives with carrier proteins (3).

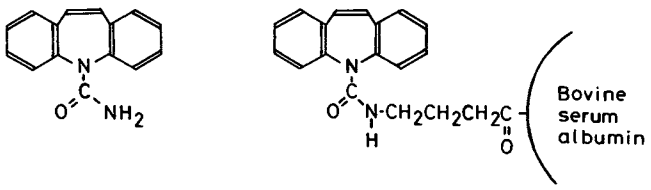

Fig. 2 Chemical structures of carbamazepine and the synthesized immunogen (carbamazepine butyryl-bovine serum albumin).

Precipitation is a reaction between a soluble antigen and a soluble antibody to yield an insoluble immunocomplex. Immunoprecipitation should be differentiated from sediment formation and from agglutination. Antibody molecules show a basic Y-shaped structure and carry at least two binding sites. If the antigen is also multivalent, the antigen forms a lattice complex or an interlocking aggregate with the antibodies, which forms insoluble particles. The particle is usually greater than 0.4 μm in diameter, consists of many antigens and antibodies, and scatters the light. The particles suspended in aqueous solution can be quantitated by nephelometry or by turbidmetry. The drug molecule is usually a monovalent antigen because of its small size and does not form a lattice complex, but forms a soluble immunocomplex. However, if more than two drug moieties are conjugated with a carrier protein, the conjugate reacts with anti-drug antibodies and forms an insoluble lattice complex (Fig. 1). Such a conjugate is called polyhaptenic antigen or developer antigen.

In precipitation inhibition immunoassay of drug, the test sample is mixed with a limited amount of synthesized polyhaptenic antigens and a limited amount of antibodies. Drug molecules in the test sample compete with the polyhaptenic antigens for the binding sites of the antibodies. Thus, the free drug inhibits the precipitation between antibodies and polyhaptenic antigens. The drug concentration inversely correlates to the precipitate formation, and can be determined by the measurement of precipitation inhibition (Fig. 3). Nephelometric immunoassay had become widely used in clinical laboratories for the quantitation of proteins such as immunoglobulins, α_1-antitrypsin and transferrin in serum before nephelometric immunoassay of drugs was developed by us (4, 5, 6). To distinguish this drug assay system from the conventional nephelometric immunoassay for proteins, we called it competitive nephelometric immunoassay. Other terms include nephelometric inhibition immunoassay or indirect nephelometric immunoassay.

Reagents: 1. Anti-Drug Antibody

Drug-specific antibody for this immunoassay is obtained by conventional methods (3). An animal such as rabbit, goat, sheep, and mouse is immunized with a drug-protein conjugate. As a carrier protein, serum albumin, serum globulin, thyroglobulin, ferritin, keyhole limpet hemocyanin, and most frequently bovine serum albumin are used. The optimal drug loading appears to be 10 to 25 hapten molecules incorporated into serum albumin. Most antibodies generated in this fashion

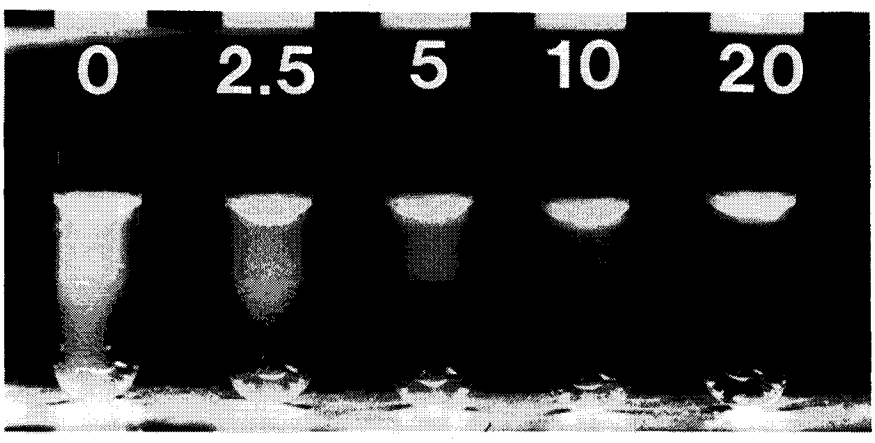

Fig. 3 Assay solutions for carbamazepine standard curve preparation. The Arabic numerals show the individual carbamazepine concentration in serum (μg/ml).

bind the hapten reversibly with association constants of 10^6 to 10^9 liters/mole. However, very small haptens are incomplete antigenic determinants which limits their sensitivity. For example, ethosuximide ($C_7H_{11}NO_2$, mol. wt. 141) and valproic acid ($C_8H_{16}O_2$, mol. wt. 143) are very small drugs and can be recognized as haptenic antigens, but they display weak affinities for their antibodies.

The selection of drug derivatives for protein conjugation must be carefully made, because the specificity or cross-reactivity of the antibodies mainly depends on the nature and location of the drug-protein link. A drug-protein conjugate induces the production of antibodies directed to various antigenic determinants. Antibodies that are directed to the spacer bridge moiety as well as the drug moiety are present in the antiserum. For example, N-carboxypropyl-carbamazepine (i.e. carbamazepine butyric acid) and N-methyl-carbamazepine show stronger affinities than carbamazepine, if an N-alkylated carbamazepine-protein conjugate is used for immunization. We can frequently predict antibody specificity by knowing the site of conjugation. Attachment of the drug-protein link should be avoided at sites of the drug molecule that undergo metabolic alteration, since the corresponding antibodies would fail to distinguish between parent drug and metabolite. It should be noted that antibodies directed solely to the carrier protein are also present in the antiserum.

The antisera are stored at 4° C after addition of 10% NaN_3 (final concentration 0.1%) and were found to be stable for at least two years in our laboratory.

Reagents: 2. Polyhaptenic Antigen

Polyhaptenic antigen may be prepared by the same method as that for immunogen preparation. However, the carrier of haptenic antigen should be different from the carrier of immunogen. If the carrier is the same, precipitation between the anti-carrier antibody and the polyhaptenic antigen would occur and interfere with the drug assay. If a rabbit is immunized with a drug-bovine serum albumin conjugate, rabbit serum albumin is recommended as the carrier of a polyhaptenic antigen, because the rabbit would not produce antibodies directed against rabbit serum albumin. We have used rabbit or human serum albumin as a polyhaptenic antigen carriers, since they are highly soluble in water causing no turbidity, stable and readily available at a low cost. Incorporation of 18 to 25 hapten molecules into serum albumin gave good assay results (4). Apoferritin was also used by others, instead of serum albumin.

The polyhaptenic antigens are stored lyophilized and remain stable at 4° C in our laboratory.

Reagents: 3. Polyethylene Glycol and Buffer

Water-soluble, straight-chain polymers are used to enhance the immunoprecipitation, thereby increasing sensitivity and reducing analysis time and reagent amounts. Polyethylene glycol (PEG) with a molecular weight of approximately 6000 is most effective and most frequently used. PEG affects the solubility of proteins. Many proteins are insolubilized by 20% PEG in the medium, but they are soluble at or below 5%. However, the aggregated immunocomplex that remains soluble in 0% PEG solution readily precipitates in 5% PEG. A 2- to 8-fold increase of drug detection sensitivity can be expected under such conditions.

We use a phosphate buffer of pH 7.4 (1/15 M Na_2HPO_4 : 1/15 M KH_2PO_4 = 8 : 2) for dilution and dissolution.

Instrumentation: Nephelometer

Nephelometers specially designed for immunoprecipitate quantitation are available from some manufacturers. Important factors for selective and sensitive quantitation of immunoprecipitation are the wavelength of the incident light and the angle to detect the scattered light. The shorter the wavelength, the stronger is the scattered light. However, the noise or background due to relatively small particles in the antiserum and assay specimen is increased at a shorter wavelength. Commercial nephelometers employ a He-Ne laser (632.8 nm) or a tungsten lamp with a 400 to 500 nm filter as the light source. The lower the angle, the stronger is the scattered light. Commercial nephelometers are designed to detect the forward scattered light at an 5° to 70° angle.

Procedure

Procedures of the commercial assay kits based on precipitation inhibition have been designed for optimal efficiency. I will describe one procedure in detail using the carbamazepine assay as an example (Fig. 4). The entire procedure is performed at room temperature.

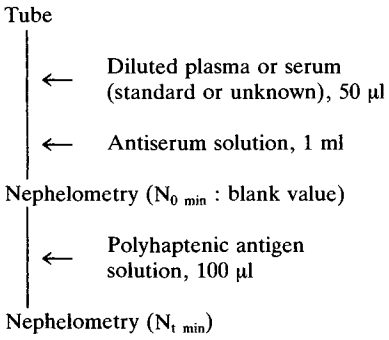

Fig. 4 Procedure of a nephelometric immunoassay in blood plasma or serum.

Step 1. Before the assay, the equivalent ratio of polyhaptenic antigen to antibody should be determined. The antiserum is diluted usually 100- to 300-fold with the phosphate buffer containing 25 g/l PEG. Then the solution is allowed to stand for more than 30 minutes during which time the solution gradually becomes turbid, and it is filtered through a membrane (pore size 0.4 µm, diameter 25 mm, Nuclepore Corp.) to remove the turbidity. Some other membranes adsorb immunoglobulins and are not recommended. In a glass tube for a laser nephelometer (Hyland Laboratories Inc.), 1 ml of the filtered antiserum solution is mixed with 100 µl of the PEG-buffer solution containing varying amounts of the polyhaptenic antigen. The precipitation occurs rapidly and is monitored on the nephelometer. With increasing amounts of the polyhaptenic antigen, increasing quantity of precipitate is formed until a maximum is reached. With further increasing amounts of the antigen, decreasing quantity of precipitate is formed. This phenomenon is called zone phenomenon, giving symmetrical precipitation curves on a semi-log graph. The zones are called antibody excess zone, equivalent zone, and antigen excess zone, respectively. If the ratio of added polyhaptenic antigen to antibodies is not optimum, the sensitivity for drug detection is lowered, because less precipitate is formed in the absence of drug and the inhibition is less efficient in the presence of drug.

Step 2. The standard plasma solutions of the drug and unknown samples are suitably diluted with a buffer.
Step 3. To 50 µl of the diluted standard or unknown is added 1 ml of the antiserum solution.
Step 4. Blank value ($N_{0\ min}$) is read on the nephelometer.
Step 5. Polyhaptenic antigen solution (100 µl) is added, the optimum dose of which has been determined as described above.
Step 6. Precipitation occurs, and the test value at t minutes ($N_{t\ min}$) is read.
Step 7. A standard curve is constructed by plotting the drug concentration vs ($N_{t\ min} - N_{0\ min}$) on a semi-log or log-logit graph.
Step 8. All unknowns are estimated by interpolation from the standard curve.

For the carbamazepine assay (6), the antiserum was diluted 150-fold, the equivalent dose of the polyhaptenic antigen was 3.2 µg in 100 µl, and plasma specimen was diluted 8-fold. The blank value was negligible except with grossly lipidemic plasma. The precipitation occurs rapidly (Fig. 5). $N_{20\ min}$ was read, and the standard curve was linear on a log-logit graph (Fig. 6). If the antiserum is diluted 300-fold instead of 150-fold, the equivalent dose of the polyhaptenic antigen is reduced to one half, and the same relative inhibition occurs at a 3- to 4-fold lower concentration of drug.

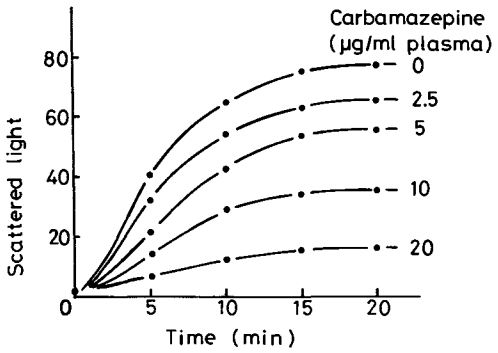

Fig. 5 Time course of the immunoprecipitation reaction and its inhibition by carbamazepine.

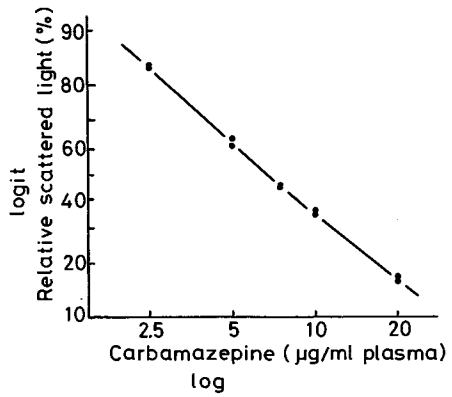

Fig. 6 Standard curve for the plasma carbamazepine assay. Relative scattered light ($N/N_0 \times 100\%$) is plotted against plasma carbamazepine concentration: $N = (N_{20\ min} - N_{0\ min})$ with each drug concentration, $N_0 = (N_{20\ min} - N_{0\ min})$ with no drug present.

Variations of the Nephelometric Immunoassay

1. Other Body Fluids. Saliva and cerebrospinal fluid are sometimes analyzed for therapeutic drug level monitoring. Because these fluids contain less light scattering material than plasma or serum, at least a five times larger volume of these fluids can be added to an assay solution. Therefore, a higher sensitivity is expected. Some of these specimens, however, require centrifugation before analysis.

2. Nephelometry and Turbidmetry. Turbidmetry is also sensitive for immunoprecipitate quantitation. Turbidmetry has the advantage of general availability of the required equipment (spectrophotometer). Turbidmetry of immunoprecipitation is performed at a shorter wavelength, usually at 340 nm or 314 nm. Our nephelometric assay procedure is directly applicable to turbidmetric detection without any other changes of the procedure.

3. Endpoint Analysis and Rate Analysis. Most nephelometric assays employ endpoint analysis, but rate analysis can also be applied. The addition of a reagent (such as formaldehyde) that blocks further precipitation may be convenient for endpoint analysis. Kinetic measurements of precipitation can be performed on a recent automated instrument equipped with a microprocessor and a temperature controller. Speed of analysis and elimination of blank reading represent the major advantages of the kinetic methods.

4. Precipitation and Agglutination. Immunoprecipitation is caused by the reaction of multivalent soluble antigens, whereas agglutination is caused by the reaction of particulate or cellular insoluble antigens. In agglutination inhibition immunoassay of a drug, the carrier of a polyhaptenic antigen is a larger particle such as a polystyrene latex bead with approximately 1 µm diameter. Drugs are directly linked or drug-protein conjugates are linked onto the surface of the particle (7). The agglutination inhibition assay has a higher sensitivity than the precipitation inhibition assay, but its problems include nonspecific aggregation, variable influence of serum matrix, rather inferior reproducibility, and variability of particle size. Generally, the agglutination inhibition assay is suitable for serum drug concentrations ranging from 0.01 to a few µg/ml and precipitation inhibition is applicable to concentrations above 1 µg/ml. When a special additional reagent, such as rheumatoid factor and C_{1q}, is used along with the pretreatment of serum with pepsin, the agglutination inhibition assay is sufficiently sensitive for the determination of serum digoxin levels at 1 to 2 ng/ml (8). The detection mode of the agglutination reaction may be nephelometry, turbidmetry, or particle counting with the use of a blood cell counter.

There is another agglutination assay system, in which an antibody-coated particle and a polyhaptenic antigen are used as reagents.

5. Homologous and Heterologous Combinations. If different drug derivatives are used for immunogen preparation and for polyhaptenic antigen preparation, sensitivity and specificity may be increased. For example, if the antiserum against carbamazepine acetyl-bovine serum albumin is used in combination with the carbamazepine butyryl-rabbit serum albumin conjugate, better assay result may be obtained.

6. Monoclonal and Polyclonal Antibodies. The technology of the production and application of monoclonal antibodies is rapidly expanding. This technology was applied to drug immunoassays mainly to remove any assay interference caused

by the antibody's cross-reactivity. The monoclonal antibody to theophylline serves as an example. Cross-reactivity with caffeine represents a general problem with theophylline immunoassays. Consequently, a mouse hybrid cell that produced a monoclonal antibody with a dramatically reduced cross-reactivity with caffeine was selected (9). After a suitable hybrid cell is obtained, the monoclonal antibody reagent can be produced in large amounts and may cost less than the conventional polyclonal antiserum reagent. However, the difficulty remains of selecting the hybrid cell that produces the most desirable antibody. A countless number of hybrid cell clones produce an antibody against a drug such as digoxin and phenytoin, and the selection requires great efforts. The conventional antiserum contains polyclonal antibodies with different affinities, but the antibodies with the greatest affinity react preferentially in the assay solution. Unless the most suitable hybrid cell has been selected from a very large number of clones, the immunoassay using monoclonal antibodies is likely to be less sensitive.

Prospective

Nephelometric immunoassays share many features with other immunoassay methods, e.g. high specificity. However, its speed and simplicity make it particularly attractive for routine application and, therefore, commercial exploitation. Presently, Beckman Instruments Inc. supplies rate-nephelometric assay kits (10, 11), Electro-Nucleonics Inc. supplies rate-turbidmetric assay kits (12, 13, 14) and Chu-gai Pharmaceutical Co. supplies endpoint-laser nephelometric assay kits. These manufacturers use their own automated analyzers, but their reagents are interchangeable. Drugs that can be determined by these kits include the anticonvulsants (phenytoin, phenobarbital, carbamazepine, and valproic acid), the aminoglycosides (gentamicin, tobramycin, and amikacin), and theophylline. The therapeutic serum concentrations of all these drugs are above 5 μg/ml, and therefore, well above the limit of sensitivity of this method. One can expect the development of commercial kits for additional drugs and improvements in assay sensitivity by employing modified procedures.

In conclusion, nephelometric immunoassay and its variants are suitable methods for therapeutic drug level monitoring with the following features: 1. no need for radioisotopes, 2. simplicity of procedure, lack of separation steps, and ready potential for full automation, 3. specificity depending mainly on the cross-reactivity of antibody, 4. sensitivity to determine serum concentration greater than 1 μg/ml using less than 10 μl of serum, 5. speed for assaying single samples or large batches, 6. ready availability of the instrumentation, i. e., nephelometer or spectrophotometer, 7. low cost and long shelf-life of the reagents, 8. easy preparation of the reagents.

References

(1) Drug Level Monitoring – Analytical Techniques, Metabolism, and Pharmacokinetics (Sadée, W., Beelen, G. C. M.) John Wiley & Sons, 1980.
(2) The Specificity of Serologic Reactions, Revised Edition (Landsteiner, K.) Dover Press, Inc., New York, 1962.
(3) Butler, Jr., V. P. (1978) J. Pharm. Exp. Ther. 29, 103–184.
(4) Nishikawa, T., Kubo, H., Saito, M. (1979) Clin. Chim. Acta 91, 59–75.
(5) Nishikawa, T., Kubo, H., Saito, M. (1979) J. Immunol. Methods 29, 85–89.
(6) Nishikawa, T., Saito, M. (1981) J. Pharm. Dyn. 4, 77–83.
(7) Hamill, P. A., Dickinson, J. C., Frey, W. A., Geltosky, J. E., Miller, W. K. (1982) Clin. Chem. 28, 1611–1611.
(8) Collet-Cassart, D., Magnusson, C. G. M., Cambiaso, C. L., Lesne, M., Masson, P. L. (1981) Clin. Chem. 27, 1205–1209.
(9) Geltosky, J. E. (1982) Clin. Chem. 29, 1201–1201.
(10) Finley, P. R., Dye, J. A., Williams, R. J., Lichti, D. A. (1981) Clin. Chem. 27, 405–409.
(11) Deaton, D., Derry, C., Decker, M. (1982) Clin. Chem. 28, 1611–1611.
(12) Wu, J. W., Hoskin, S., Riebe, S. M., Gifford, J. E., O'Neill, S. P. (1982) Clin. Chem. 28, 659–661.
(13) Dugan, S., Bailey, J., Wu, J. W., Hoskin, S., Riebe, S., Gifford, J., O'Neill, S. P. (1982) Clin. Chem. 28, 1190–1191.
(14) Wu, J. W., Bunyagidj, C., Hoskin, S., Riebe, S. M., Aucker, J., White, K., O'Neill, S. P. (1983) Clin. Chem. 29, 1540–1542.